Growth factor and receptor modulations in rat liver by choline-methionine deficiency

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Introduction

The essential roles of dietary choline in maintaining proper cellular and biological functions of various organs have been well characterized.[1,2] Thus, it is not surprising to observe that its lack in the diet, resulting in a state of methyl deficiency, induces a variety of anatomical lesions in many organs of a wide variety of experimental animals.[3] The deficiency state also alters the responses of animals to various exogenous stimuli. One of the most extensively studied areas of modified host responses to external stimuli in methyl deficiency is its effects on processes of cancer development. It has indeed become evident from the results of many experimental studies conducted during the past 50 years that a choline-methionine (methyl-deficient) diet acts not only as a complete carcinogen in several species of animals, but also as a strong cocarcinogen and an efficient tumor promoter.[3,4,5] Even though the carcinogenicity of a methyl-deficient diet has been demonstrated in many organs, such as liver, pancreas, lung, and urinary bladder, except for the liver, its carcinogenicity is questionable.[4] The target organs of the cocarcinogenic and promoting effects of the diet are limited to the liver with a few exceptions.[6]

Many of the pathological lesions caused by choline deficiency are the consequence of disturbances of three major biological functions of choline. It is an essential component of phospholipids, it serves as a methyl donor in transmethylation reactions, and it is a precursor of acetyl-choline. The roles of a modified methylation state of cellular macromolecules in the pathogenesis of various lesions induced by choline-methionine deficiency, including modified carcinogenesis, have been discussed.[7] Disturbances of phospholipid metabolism in cells lead to structural and functional alterations of cell membranes. For the past few years, the major thrust of investigation in our laboratory has been to elucidate the significance and relevance of these membrane alterations to the process of liver carcinogenesis.[8,9] The structural, biochemical, or functional alterations of the membranes can indeed lead to disturbances of a number of metabolic steps involved in cellular signal transduction pathways (*Figure 1*). Normal cell growth is controlled by orderly sequences of signal transduction, and disturbances of any one step in the cascade of the reaction may lead to aberrant cell growth relevant to tumor promotion and/or carcinogenesis.

In the present paper, we briefly review the existence of several growth factors that regulate liver cell growth and the evidence that alterations in the cell membrane receptors of some of these growth factors occur in the liver of rats fed a choline-deficient (CD) diet. Recent studies on modulations of growth factor production during the early and tumor-induction stages of choline deficiency then will be presented. The major emphasis will be placed on hepatocyte growth factor (HGF), a recently characterized potent growth factor for hepatocytes, and on two other well-known factors, transforming growth factor α(TGF-α) and transforming growth factor-β1 (TGF-β1).

Growth factors involved in the regulation of hepatocyte growth

Studies with isolated hepatocytes and hepatocyte cultures maintained in serum-free media have been used extensively to identify stimuli and signals involved in the induction of hepatocyte proliferation. From these studies, it has become apparent that a large number of agents affect hepatocyte growth in vitro. These agents can be classified into two major types: complete hepatocyte mitogens that recruit quiescent hepatocytes from nonproliferating conditions to fully proliferative responses in the absence of any potential growth stimulators; and comitogens that are not mitogenic in serum-free conditions, but enhance hepatocyte proliferation

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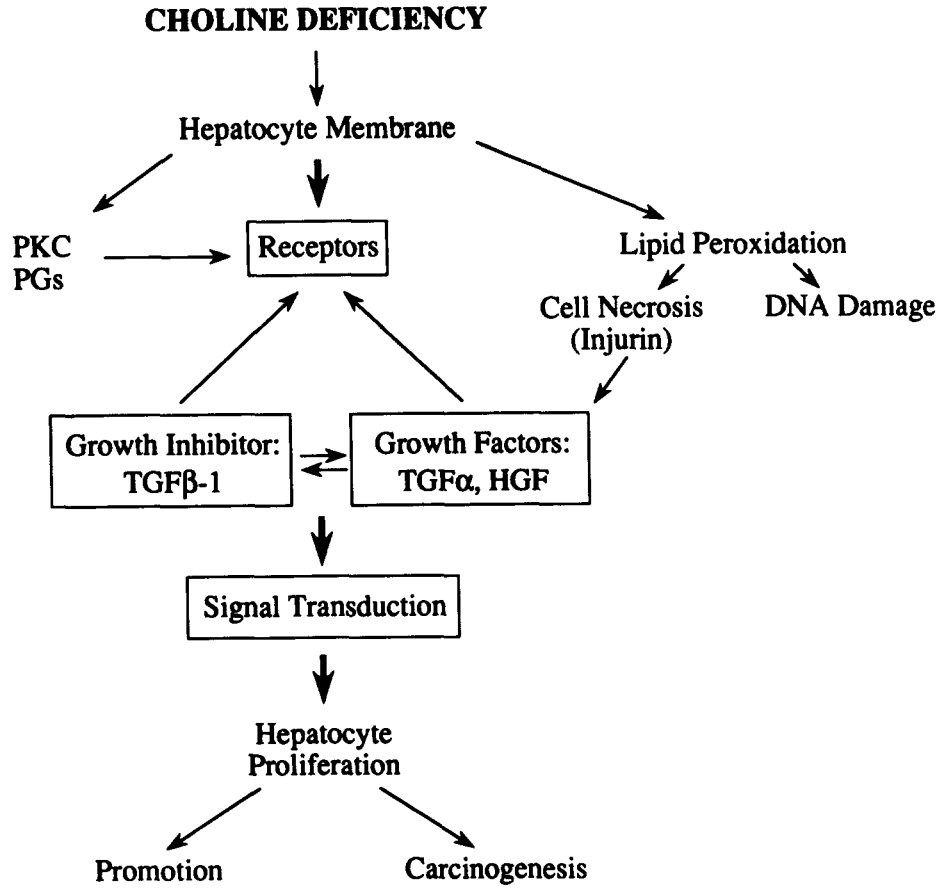

Figure 1 Schematic illustration of possible consequences of hepatocyte membrane alterations resulting from choline deficiency. PKC, protein kinase C; PGs, prostaglandins.

induced by other growth factors (*Table 1*).[10] Epidermal growth factor (EGF), originally described by Cohen,[11] is a mitogenic peptide affecting a variety of cells,[12] and was the first agent shown to stimulate hepatocyte DNA synthesis in serum-free chemically defined conditions.[13] The protooncogene, erbB, is the receptor of EGF that possesses intrinsic protein tyrosine kinase.[14] TGF-α was first described in the condition medium of certain retrovirus transformed tumor cells.[15] TGF-α and EGF share the same receptor, and TGF-α exerts a stronger mitogenic effect than EGF.[10] Acidic fibroblast growth factor also stimulates DNA synthesis on a certain specific subpopulation of hepatocytes in the presence of heparin.[16] HGF is a recently identified and characterized mitogen for adult hepatocytes in culture.[17,18] It is, however, well established by now that HGF exerts multiple functions in addition to its mitogenic action on hepatocytes; it controls the motility of cells as a "scatter factor",[19] and it is involved in the morphological differentiation of tissue structures as a "morphogen".[20] It is the most potent direct mitogen for hepatocytes. As in the case of other growth factors like EGF and TGF-α, HGF initiates its effect by binding to cell surface receptors that were shown to be the products of the protooncogene, c-Met.[21]

TGF-β is a family of multifunctional regulators of cell growth and differentiation for a wide variety of cells.[22] It was initially identified as a factor that stimulates the anchorage-independent growth of rodent fibroblasts.[23] In contrast to the growth factors mentioned above, TGF-β1 inhibits hepatocyte growth in culture[24,25] and perhaps in vivo.[26] It also inhibits hepatocyte proliferation induced by EGF, TGF-α and HGF. Support for the notion that these factors exert either positive or negative stimuli for hepatocyte proliferation in vivo came also from observations that the time course of appearance of these factors in the blood circulation and of expression of their genes in the liver coincides with the time course of the induction and cessation of liver cell proliferation in several models of liver regeneration.[27-31]

Table 1 Growth factors for hepatocyte proliferation

Direct mitogens	Comitogens
Epidermal growth factor (EGF)	Insulin
Transforming growth factor-α (TGF-α)	Glucagon
Hepatocyte growth factor (HGF)	Norepinephrin
Acidic fibroblast growth factor (α-FGF)	Vasopressin
Hepatocyte stimulatory substance (HSS)	Angiotensin II
Tumor necrosis factor-α (TNF-α)	

Substances termed as comitogens are not themselves

direct mitogens, but exert profound effects in modulating the responses to direct mitogenic or mito-inhibitory factors. Insulin and glucagon are classic examples of comitogens, and both enhance the mitogenic actions of EGF, TGF-α, and HGF.[13,32] It is well known that the actions of these polypeptide hormones are mediated by bindings to specific receptors. In addition to these well-characterized hepatocyte mitogens and comitogens, another substance called hepatocyte stimulatory substance (HSS) has recently been described as a mitogen for hepatocytes in vivo.[33–35] HSS is extracted from neonate and regenerating livers, but its precise molecular structure and mechanism of action have not yet been determined. Recent studies by several investigators suggest that a multifunctional cytokine, tumor necrosis factor-α (TNF-α) also has a mitogenic effect on hepatocytes both in vivo[36–38] and in vitro.[39] It remains to be clarified whether TNF-α acts as a direct mitogen or a comitogen.

Hepatocyte membrane receptor alterations induced by a CD diet

Cellular metabolism, growth, and differentiation are modulated by various hormones and growth factors, and, thus, alterations in cell membrane receptors for these factors may lead to aberrant cell growth and ultimately to cell transformation. In an initial attempt to determine whether a CD (both the CD and choline-supplemented diets were prepared by Dyets, Inc., Bethlehem, PA USA, and their compositions have been described.[40]) diet induces hepatocyte surface receptor alterations, we selected insulin as a ligand. Hepatocytes isolated by collagenase perfusion and binding of ^{125}I-labeled insulin were used in the assays. The competition curves and Scatchard plots of control hepatocytes and of hepatocytes of rats fed a CD diet for 7–14 days showed a marked decline in insulin receptor number and an increased binding affinity (decreased K_d) in CD hepatocytes.[41] Hepatoma cells derived from liver tumors induced by a CD diet containing ethionine showed further declines in both of the features of insulin receptors.[39] The questions were then addressed as to whether liver tumor promoters other than a CD diet also induced a similar change in hepatocyte insulin receptors, and as to whether alterations such as those observed with insulin receptors occurred also in other peptide hormone receptors. The results of these studies showed that phenobarbital, a prototype experimental liver tumor promoter, also induced decreases in the number of hepatocyte insulin receptors.[42] Neither a CD diet nor phenobarbital had any effect on the number of glucagon receptors or the binding affinity.[42] However, the two promoters both induced changes in the levels and binding affinity of EGF similar to those observed in the case of the insulin receptors.[43] Progressive decreases of EGF bindings in normal hepatocytes, preneoplastic hepatocytes, and hepatoma cells during hepatocarcinogenesis have also been reported.[44] The overall findings on hepatocyte receptor alterations induced by a CD diet and phenobarbital are summarized in *Table 2*. As can be seen, both a CD diet and phenobarbital induced changes in insulin and EGF receptors, but their effects on glucagon receptors were negligible. The insulin and EGF receptors are both associated with tyrosine kinase activity and autophosphorylated preferentially at the tyrosine residues upon ligand-receptor interaction.[45,46] Glucagon receptor-mediated actions instead involve activation of the adenyl cyclase-cyclic AMP system.[47] Therefore, the confinement of alterations to receptors with endogenous tyrosine kinase activity induced by a CD diet suggests that the diet induces selective disturbances in many pathways involved in cellular signal transduction. The concept that a progressive disorder in signal transduction plays a critical role in the mechanism of action of chemical carcinogens and of tumor promoters has been considered.[48] As already discussed, several well-defined growth factors have been identified, many of which exert their effects through receptor-mediated events. It is conceivable, therefore, that changes in the production of one or more of the growth factors and/or alterations of their receptors could lead to disturbances of the homeostatic growth of liver cells.

Changes in gene expression of selected liver cell growth factors

An enhanced liver DNA synthesis and liver cell proliferation have been suggested as playing important roles in the processes of CD diet-mediated liver tumor production and carcinogenesis.[49,50] The critical role that enhanced cell proliferation may have in the genesis of both human and experimental cancers has been reiterated in recent years.[51–53] As discussed earlier, several growth factors that trigger liver cell proliferation in vivo have been characterized. It has become apparent also that liver cell proliferation in vivo is controlled by an intricate interplay of both stimulatory and inhibitory stimuli provided by these growth factors. The observation that a CD diet induces hepatocyte membrane receptor alterations suggested that it may also modify the production of these growth factors. To test this possibility, we analyzed the steady state levels of mRNA for HGF, TGF-α, and TGF-β1 in the liver of rats fed a CD diet. Expression of c-Met, the receptor for HGF, was also examined.

Early stages of choline deficiency

Male F344 rats were fed a CD or a choline-supplemented (CS) diet. Poly (A)+ RNA was prepared from livers 1, 2, 4, and 6 weeks thereafter. RNAs were electrophoresed in an agarose formaldehyde gel, transferred to nylon membranes, and hybridized with ^{32}P-labeled cDNA probe for HGF, TGF-α, TGF-β1 and c-Met.[54,55] *Figures 2 and 3* illustrate selected Northern blots of HGF, TGF-α and TGF-β1. There were gradual increases in the levels of HGF and TGF-α mRNA in the liver of rats fed a CD diet beginning 2 weeks after the start of feeding. Elevated levels of TGF-β1 mRNA were already evident in the CD rats at 1 week, and persisted for 6 weeks (*Figure 4*). The level of HGF expression steadily increased during the 6-week feeding

Table 2 Hepatocyte membrane receptor alterations by choline deficiency

Treatment	Cell surface receptors					
	Insulin		Glucagon		EGF	
	No.	K_d	No.	K_d	No.	K_d
Choline deficient diet	↓	↓	—	—	↓	↓
Phenobarbital	↓	↓	—	—	↓	↓

—, No change; ↓, decrease.
Hepatocytes were isolated by collagenase perfusion from the livers of 3–4 male Sprague Dawley rats fed a choline-deficient diet for 2–3 weeks and a basal diet containing 0.06% phenobarbital for 4–5 weeks. Bindings of ^{125}I-labeled ligands were used in the assays.[41–43]

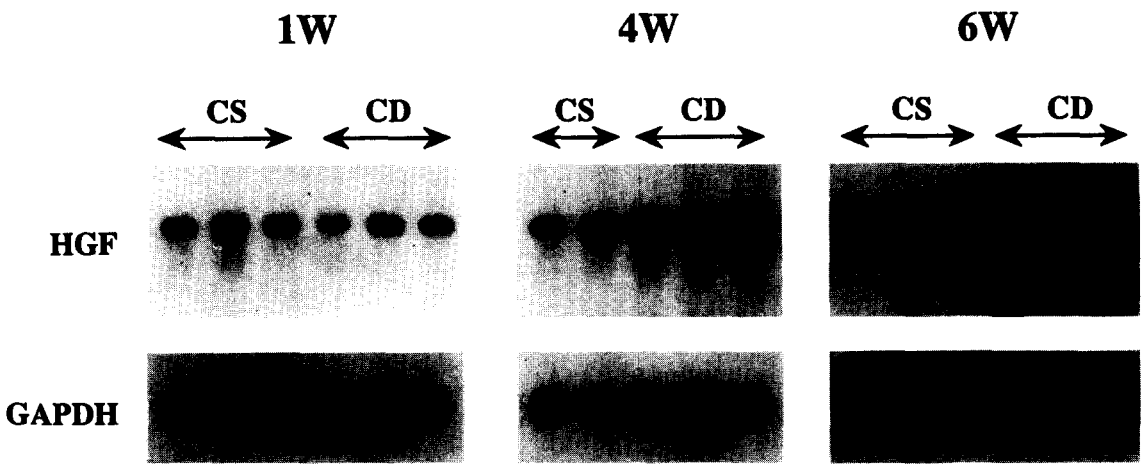

Figure 2 Northern blot of HGF mRNA from the liver of rats fed a choline-supplemented (CS) or choline-deficient (CD) diet for 1, 4, and 6 weeks. Six μg of each poly(A)$^+$ RNA was electrophoresed in an agarose-formaldehyde gel, transferred to nylon membrane, and hybridized with ^{32}P-labeled 1.4Kb HGF cDNA probe. The expression of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), is also shown.

of the CD diet, but the level of C-Met mRNA was no different between the two dietary groups (*Figure 5*). As in the case of the EGF and insulin receptors, which were selectively altered by choline-deficiency, the HGF receptor is also a member of the tyrosine kinase receptor family.[21] Thus, it would be interesting to examine possible alterations of hepatocyte HGF receptors during the course of CD diet feeding.

Stages of hepatoma development

To assess the significance of these early changes, we extended our studies to later stages of carcinogenesis. For this study, male F344 rats were initiated with a single IP dose of diethylnitrosamine (250 mg/Kg body weight), followed by feeding a CS or CD diet for 7–8 months. Northern blot analyses of mRNAs from hepatomas and nontumorous areas of hepatoma-bearing livers from three rats and from the liver of two rats each fed a CS or basal diet were performed. *Figure 6* illustrates changes in the expression of HGF and c-Met, and *Figure 7* of TGF-α and TGF-β1. It is evident that hepatocellular carcinomas induced by the experimental regimens did not produce HGF, but they express c-Met mRNA suggesting that the hepatoma cells possess HGF receptors. These findings are consistent with those showing that the source of HGF production in the liver are nonparenchymal cells such as Kupffer cells, endothelial cells, and Ito cells.[56,57] Hepatomas may not contain significant numbers of these HGF-producing cells. Marked elevations of HGF mRNA were seen in nontumorous areas of CD-treated livers, suggesting that HGF may stimulate the growth of hepatocellular carcinomas through a paracrine mechanism. The findings are of considerable interest because there are several reports indicating that HGF inhibits the growth of hepatocellular carcinoma cell lines maintained in culture.[58,59] Thus, the effects in vivo of HGF on hepatoma development may be different from its effects seen in system in vitro.

High levels of expression of TGF-α and TGF-β1 were noted in hepatocellular carcinomas as well as in the adjacent nontumorous liver of hepatoma-bearing rats (*Figure 7*). TGF-α, in addition to HGF, may contribute to growth in vivo of hepatocellular carcinoma through both paracrine and autocrine mechanisms. The production of TGF-α by hepatocellular carcinomas has been reported.[60] Because TGF-α and EGF share common receptors, the decreased number of EGF receptors frequently observed in hepatoma cells[44] may be a consequence of over production of TGF-α by nontumorous liver as well as by hepatoma cells down regulating the EGF receptors. The significance of elevated TGF-1 expression in hepato-

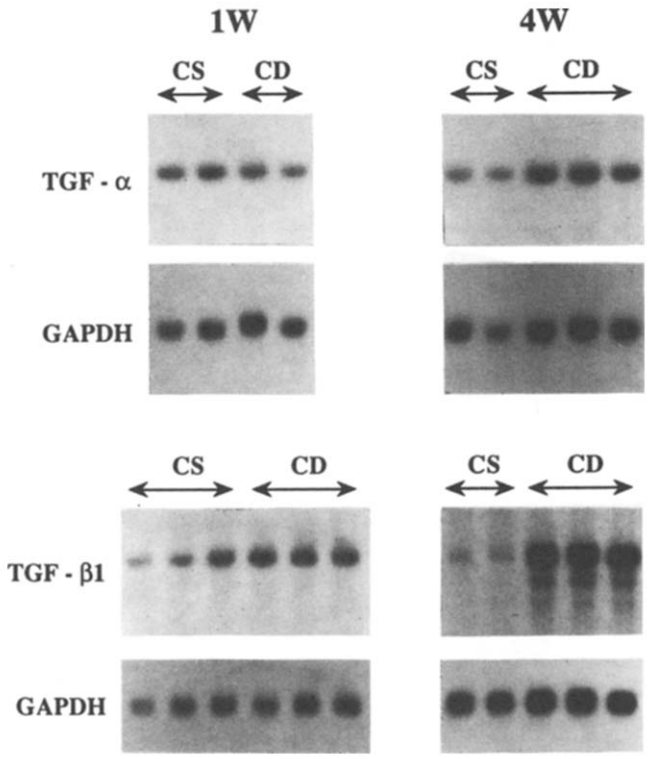

Figure 3 Northern blot of TGF-α and TGFβ-1 mRNAs from the liver of rats fed a CS or CD diet for 1 and 4 weeks. A partial cDNA of TGF-α prepared by RNA polymerase chain reaction[54] and a 0.75 Kb KpnI-EcoRI fragment of λTβC, TGF-β cDNA (a gift from R. Derynck, Genetech, San Francisco, CA) were used as probes.

mas and nontumorous liver of rats fed a CD diet is, however, unclear. Similar elevations of TGF-β1 expression were observed in liver carcinogenesis induced by a CD diet containing ethionine, and the possibilities were considered that carcinogen-initiated hepatocytes and hepatoma cells become refractory to the inhibitory effects that TGF-β1 has on the growth of normal hepatocytes.[61] Because hepatoma cells apparently have the same type of TGF-β1 receptors as normal hepatocytes, the loss of sensitivity to TGF-β1 in hepatoma cells was considered to be caused by a defect occurring past receptor mechanisms.[61] The liver of hepatoma-bearing rats fed the CD diet showed complex histological alterations characterized by considerable fibrosis, chronic inflammatory cell infiltration, and hepatocyte regenerative activity. Because TGF-β is known to exert its effect on inflammation and repair and on lymphocyte functions,[22,62] it is conceivable that the primary effects of TGF-β1 during the course of hepatoma induction may be directed toward enhanced collagen synthesis and modulations of host immune functions.[62] Lipotrope deficiency is known to induce disturbances of host immune functions.[63] TGF-β1 plays multiple roles in the liver of rats fed a CD diet.

Summary and conclusions

One of the major pathological lesions resulting from choline-methionine deficiency is hepatocyte membrane alterations, which lead to further disturbances in several pathways of cellular signal transduction. Hepatocyte proliferation induced by a CD diet may play a critical role in diet-induced tumor promotion and carcinogenesis. In the present paper, we reviewed the existence of several known growth factors that are involved in the regulation of liver cell growth, and the evidence that a CD diet induces selective changes in the membrane receptors of some of these growth factors. Recent data on modulations of growth factor production in the liver of rats fed a CD diet, as viewed by the levels of mRNA expression in the diet-treated liver and in induced hepatocellular carcinomas, were presented. During the course of hepatoma induction, CD-diet promotion is accompanied by elevated levels of HGF, TGF-α, and TGF-β1 mRNA. HGF mRNAs were highly expressed in the nontumorous areas of the liver adjacent to hepa-

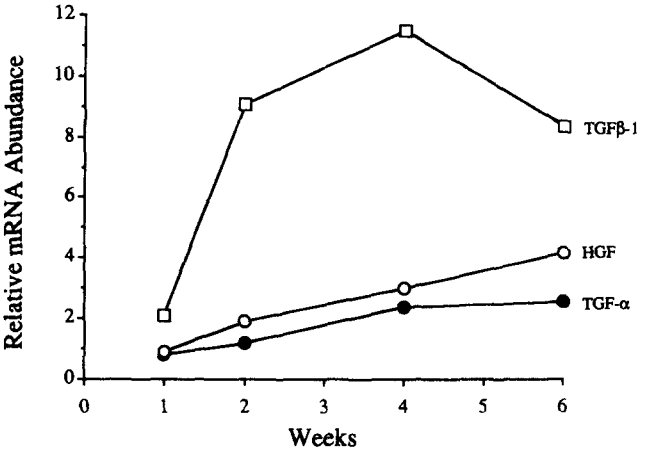

Figure 4 Relative abundance of mRNAs for HGF, TGF-α, and TGF-β1 in the liver of rats fed a CS or CD diet. Three rats were used at each time point for the assay. Relative abundance of specific transcripts in the different lanes was determined by densitometric analyses of the autoradiographs and was expressed as "fold increase" of specific mRNA by calculating the mean values of the abundance of mRNA in rats fed the CD diet to the corresponding values of mRNA in rats fed the CS diet.

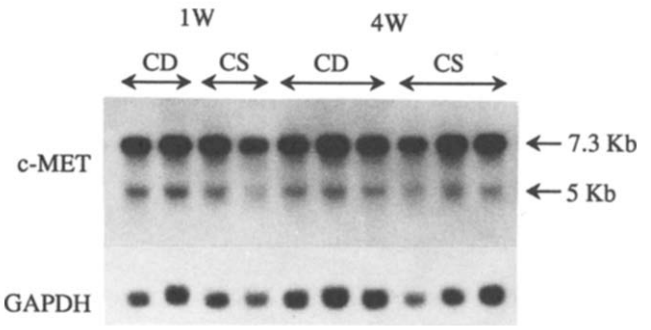

Figure 5 Northern blot of c-Met mRNA from the liver of rats fed a CS or CD diet for 1 and 4 weeks. A 0.467 Kb c-Met cDNA probe was prepared by RNA polymerase chain reaction as previously described.[55]

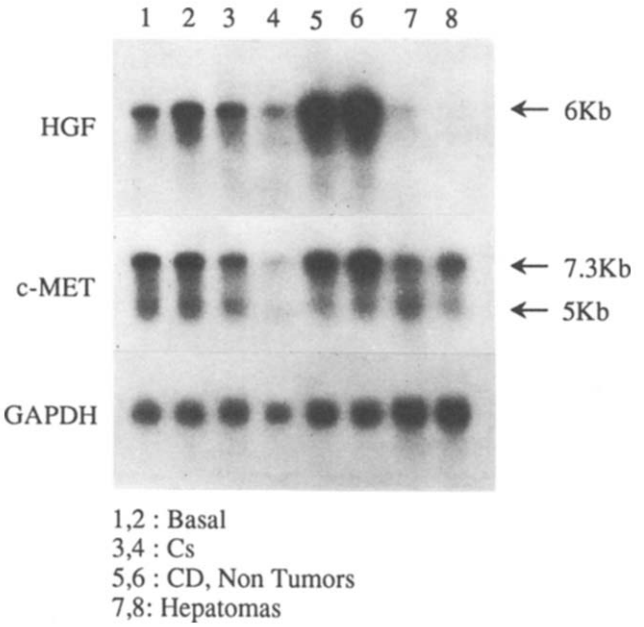

Figure 6 Northern blot of HGF and c-Met mRNAs from the liver of rats fed a basal (lanes 1 and 2) or CS (lanes 3 and 4) diet, non-tumorous areas of hepatoma-bearing liver (lanes 5 and 6) and hepatomas (lanes 7 and 8) induced by a single dose of diethylnitrosamine (DEN) followed by 7–8 months CD diet.

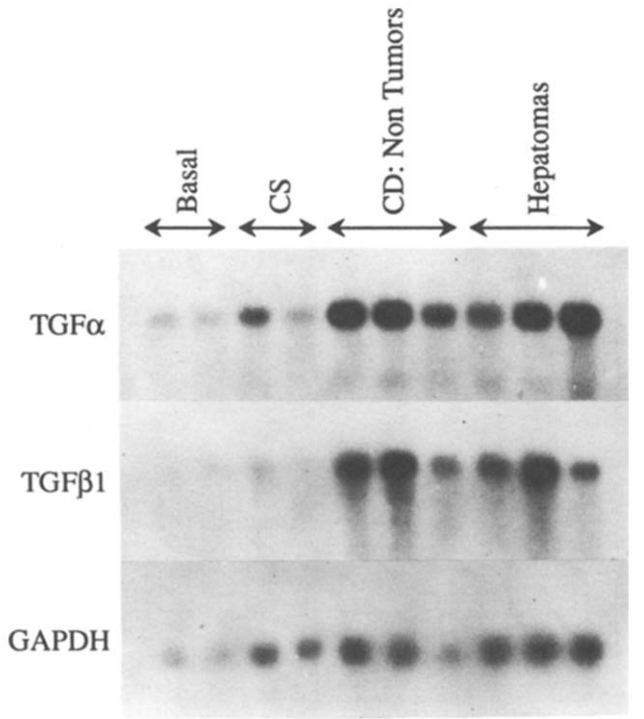

Figure 7 Northern blot of TGF-α and TGF-β1 mRNAs from the livers of control rats (Basal and CS) and rats treated with a single dose of DEN followed by continuous feeding of a CD diet.

tocellular carcinomas, which themselves showed no expression of HGF mRNA. Thus, HGF appears to stimulate hepatoma growth by a paracrine mechanism. Elevated levels of TGF-α mRNA in both hepatomas and in the nontumorous areas of the liver suggest that TGF-α stimulates tumor growth by both paracrine and autocrine mechanisms. Elevated levels of TGF-β1 in the liver during the course of tumor production and in hepatocellular carcinomas may reflect the multi-functional nature of this growth factor, which is involved in the development of various pathological lesions in the liver. A long list of growth factors, both direct and comitogenic to hepatocytes, is now available, and more will be discovered and characterized in the future. Interplay of these multiple factors, rather than any one specific factor, play critical roles in regulating hepatocyte proliferation, and an imbalance of actions of these factors induced by a CD diet may be one of the contributing factors for the tumor promoting and/or carcinogenic effects of the diet.

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